



Full length article

Balanced role of T3SS and T6SS in contribution to the full virulence of *Edwardsiella piscicida*

Tianjian Hu^a, Ran Chen^a, Lingzhi Zhang^a, Zhuang Wang^a, Dahai Yang^{a,b}, Yuanxing Zhang^{a,b,c}, Xiaohong Liu^{a,b,**}, Qin Liu^{a,b,c,*}

^a State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China

^b Shanghai Engineering Research Center of Maricultured Animal Vaccines, Shanghai, 200237, China

^c Shanghai Collaborative Innovation Center for Bio-manufacturing, Shanghai, 200237, China

ARTICLE INFO

Keywords:

Edwardsiella piscicida
Infection
Live attenuated vaccine
T3SS
T6SS

ABSTRACT

Edwardsiella piscicida is an important pathogen that infects a wide range of hosts, from fish to human. Its infection leads to extensive losses in a diverse array of commercially important fish, like Japanese flounder, turbot, and tilapia. During the infection, type III secretion system (T3SS) and type VI secretion system (T6SS) of *E. piscicida* play significant roles, but how T3SS and T6SS cooperatively contribute to its virulence is still unknown. In this study, we first examined the roles of T3SS and T6SS in different processes during *E. piscicida* infection of host cells, and revealed that T3SS of *E. piscicida* is responsible for promoting bacterial invasion, the following intracellular replication and inducing cell death in host cells, while T6SS restrains *E. piscicida* intracellular replication and cell death in J774A.1 cells, which suggested that T3SS and T6SS antagonistically concert *E. piscicida* infection. Furthermore, we found a significant decrease in transcription level of IL-1 β in zebrafish kidney infected with T3SS mutant and a drastically increase in transcription level of TNF- α infected with T6SS mutant when compared with the wild-type. Interestingly, both T3SS and T6SS mutants showed significant attenuated virulence in the zebrafish infection model when compared with the wild-type. Finally, considering the cooperative role of T3SS and T6SS, we generated a mutant strain WED Δ T6SS based on the existing live attenuated vaccine (LAV) WED which showed improved vaccine safety and comparable immune protection. Therefore, WED Δ T6SS could be used as an optimized LAV in the future. Taken together, this work suggested a bilateral role of T3SS and T6SS which respectively act as spear and shield during *E. piscicida* infection, together contribute to *E. piscicida* virulence.

1. Introduction

Bacteria have evolved specialized nanomachines with the remarkable ability to inject multiple bacterially encoded effector proteins into eukaryotic or prokaryotic cells. Known as type 1–9 secretion systems, these machines play a central role in the pathogenic or symbiotic interactions between multiple bacteria and their eukaryotic hosts [1,2]. In many pathogens, more than one secretion system have been discovered and well-studied, which collaboratively contribute to bacteria virulence, and among these secretion systems, type III secretion system (T3SS) and type VI secretion system (T6SS) are most studied [3–8]. For *Salmonella*, T3SS is important for bacterial internalization and intracellular survival, and is crucial for bacterial infection in mice [3,4], while T6SS has been implicated in biofilm formation, cytotoxicity,

invasion, and survival within macrophages [5,6]. For *E. coli*, T3SS contributes to bacterial intracellular survival in chicken macrophages, and is involved in bacterial colonization and survival during infection *in vivo* [7], meanwhile T6SS is related to bacterial adhesion and invasion in epithelial cells, and bacterial replication within macrophages [8]. A deep insight into the role of T3SS and T6SS involving in bacterial infection is the most important aspect for better understanding of bacterial pathogenesis.

Edwardsiella piscicida is a Gram-negative, facultative anaerobe, and short rod-shaped microorganism, and it's an important intracellular pathogen causing systemic infection in a wide variety of marine and freshwater fish, and infects other hosts, ranging from birds and reptiles, to mammals [9,10]. In *E. piscicida*, T3SS and T6SS are identified as two of the most important virulence mechanisms [11,12]. The proteins

* Corresponding author. State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200237, China.

** Corresponding author. Shanghai Engineering Research Center of Maricultured Animal Vaccines, Shanghai, 200237, China.

E-mail addresses: qinliu@ecust.edu.cn (X. Liu), liuxiaohong@ecust.edu.cn (Q. Liu).

Table 1
Primers used in this study.

Name	Sequence (5'-3')
β -actin: F	ATGGATGAGGAAATCGCTGCC
β -actin: R	CTCCCTGATGTCTGGGTCTGTC
IL-1 β : F	TGGACTTCGCAGCACAAAATG
IL-1 β : R	CACCTTCACGCTCTTGGATGA
TNF- α : F	AAGGAGAGTTGCCTTTACCG
TNF- α : R	ATTGCCCTGGGTCTTATGG
IL-6: F	TCAACTTCTCCAGCGTGATG
IL-6: R	TCTTTCCCTCTTTTCTCTCTG
IL-8: F	CCCTGTGACACTCAAGAGCT
IL-8: R	CAGTAGCCTTACCCATGGA
IL-10: F	TCACGTCATGAACGAGATCC
IL-10: R	CCTCTTGCAATTCACCATATCC
P1	GAGCTCAGGTTACCCGATGCAAGATCTATAAGATCCCGTCTATGCCT
P2	CTAAATCGGAAGTCACTCCGTAAACATTTCTTACA
P3	ATGACTTCCGATTTAGCCGAGTTTGTTTACCAG
P4	CCCTCGAGTACGCGTCACTAGTGGGGCCCTCGCCTGCGGTTCTG

EseB, EseC, EseD and EseH belonging to T3SS, are related to the translocation of effector proteins in infected host cells [11,13]. Meanwhile, proteins of T6SS, EvpA, EvpB and EvpC are associated with virulence of *E. piscicida* [14,15]. Moreover, four T3SS effectors, EseG [16], EseH [13], EseJ [17], EseK [18], and one T6SS effector, EvpP [19], have been found to play important roles in bacterial infections. In *E. piscicida*, T3SS mutant and T6SS mutant are unable to secrete corresponding secreted proteins, and they also show significantly impaired cell invasion capabilities in macrophage [20]. Tan et al. (2005) demonstrated that T3SS plays an important role in phagocyte survival and virulence, and T3SS mutant also affects *E. piscicida* adherence to and internalization into epithelial cells [14]. Furthermore, T3SS is required for intracellular replication of the bacterium in murine macrophages [21]. T6SS was recently identified and proposed to allow the bacteria to establish intracellular infection within the hosts, causing severe systemic infection, and eventually kill the hosts [11,12,22]. Previous studies demonstrated that T3SS and T6SS play essential roles during the infection of *E. piscicida* *in vivo* [22–25]. However, work on these critical virulence factor is not often brought together to create a detailed picture of *E. piscicida* infection mechanism.

Members of *Edwardsiella* are associated with animals found in freshwater and marine water environments [26]. *Edwardsiella ictaluri* (*E. ictaluri*) causes a systemic infection called enteric septicemia in channel catfish and is normally associated with this host [27]. On the other hand, *Edwardsiella hoshinae* (*E. hoshinae*) is less frequent but has a wider host distribution than *E. ictaluri* [26]. In the aquaculture industry, *E. piscicida* is the causative agent of edwardsiellosis and ascites disease, and *E. piscicida* infection causes subcutaneous bleeding and ascites appears in the abdominal cavity [12]. The clinical signs of *E. piscicida* infection are common to all species of fish suffering from the disease. Externally, affected fish show discolored areas of the skin with loss of pigmentation, external haemorrhages and a general septicemia in the ventral muscle [28,29]. Moreover, exophthalmia, abundant ascitic fluid and general petechiae in the internal organs were also observed in turbot [30]. Histological examinations performed in largemouth bass revealed multifocal necrosis scattered throughout the heart, liver, anterior kidney, posterior kidney and spleen [31]. To combat the threatening disease in fish, many candidate vaccines against edwardsiellosis have been developed and actively evaluated in clinical trials [32–35]. Among these, live attenuated strains of *E. piscicida* have generated much interest in the search for improved vaccines and as vaccine vectors for the delivery of heterologous antigens [32]. A promising vaccine candidate against edwardsiellosis has already been explored namely WED which owned its attenuation mainly due to lack of the T3SS [32]. Considering of the important role of both T3SS and T6SS, promising live attenuated vaccines may be further designed.

In this study, we aim to explore the roles of T3SS and T6SS together

in different process during *E. piscicida* infection in host cells and zebrafish. By comparing the ability of *E. piscicida* wild-type, T3SS mutant and T6SS mutant to adhere, invade and replicate inside host cells and the ability to trigger cell death, we found a balanced role of T3SS and T6SS involving in these processes. Specifically, T3SS is essential for *E. piscicida* to invade and replicate in host cells while T6SS restrains the infection. Further *in vivo* experiments showed a significant decreased bacterial burden but increased expression of TNF- α of T6SS mutant when compared to the wild-type, suggesting a positive role of T6SS involving in restricting host immune response. Both T3SS and T6SS mutants showed dramatic attenuation of virulence in zebrafish. Based on these results, we optimized the existing vaccine WED by combining WED with T6SS mutations to generate a WED Δ T6SS which retained the immune protection with improved vaccine safety. These results reveal the basic mechanism of T3SS and T6SS in *E. piscicida* infection, guiding a clue for further vaccine designation.

2. Materials and methods

2.1. Bacterial strains, cell cultures, and primers

The wild type pathogenic strain of *E. piscicida* (EIB202) was isolated from ascites of diseased turbot in Yantai (Shandong, China), Δ T3SS was constructed by deletion of *eseB-eseD*, Δ T6SS was constructed by deletion of *evpA-evpB*, and these all were completed by Yang et al. (2015) [20], the live attenuated vaccine strain WED was constructed in our previous work [32], WED Δ T6SS was constructed by deletion of *evpA-evpB* on the basis of WED, and the primers were shown in Table 1. Bacteria was grown in tryptic soy broth (TSB), tryptic soy agar (TSA), or Dulbecco's modified essential medium (DMEM) at 30 °C. HeLa cells (ATCC number CCL-2) and J774A.1 cells (ATCC number TIB-67) were cultured at 37 °C in a 5% CO₂ atmosphere in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μ g/ml).

2.2. Cell infection and enumeration of intracellular and extracellular bacteria

HeLa cells were seeded into 24-well plates with 1×10^5 cells per well and J774A.1 cells were seeded into 24-well plates for 1.5×10^5 cells per well. *E. piscicida* EIB202, Δ T3SS, and Δ T6SS were grown in TSB at 30 °C with shaking for 12 h, and then diluted (1%, v/v) into DMEM for static culture at 30 °C until the OD₆₀₀ reached 0.9. Harvested bacteria were washed with PBS 3 times and added to cells at different multiplicities of infection (MOIs). The plates were then centrifuged at 600 \times g, 30 °C for 10 min.

To enumerate the bacteria that adhere to the cell surface, after 2 h post infection, cells were washed with PBS 3 times to remove the free

bacteria. Triton X-100 was added to the cell culture at 1% (v/v). The yielded mixture of lysis was serially diluted and counted as CFU. The results were expressed as the ratio (CFUs per cell).

To enumerate intracellular bacteria, at the indicated times, cells were treated with 1000 µg/ml gentamicin for 30 min to kill the bacteria which had adhered to the cell surface, followed by washing with PBS. After washing with PBS, Triton X-100 was added to the cell culture at 1% (v/v). The yielded mixture of lysis was serially diluted and counted as CFU. The results were expressed as the ratio (CFUs per cell) of intracellular bacteria.

2.3. Cell death assay

Cells were infected as described above and supernatants were harvested at the indicated time points. Lactate dehydrogenase (LDH) release was quantified using the Cytotox96 Assay Kit (G1780, Promega) according to manufacturer's instructions. Cytotoxicity was normalized to Triton X-100 treatment (100% of control), and LDH release from uninfected/untreated cells was used for background subtraction.

2.4. Adult zebrafish challenge and sample treatment

Challenge protocol was approved by the local animal center at East China University of Science and Technology (Shanghai, China). The challenge was performed by intramuscular injection of different bacteria (100 CFU per fish). The cumulative survival rate was determined over a week period.

After challenged, five fish from each group were anesthetized and killed at indicated time. Liver, spleen, and kidney were aseptically removed and weighed to normalize the samples. Then, the organs were digested in 0.25% trypsin for 20 min, and were homogenized in 500 µl PBS. The homogenates were ground and serially diluted and plated in triplicate onto DHL plates containing 16.7 µg/ml Colistin (Col⁺), and the plates were incubated at 30 °C for 48 h. The colonies that featured with black centers were counted.

2.5. Zebrafish larval challenge

For zebrafish larvae yolk sac microinjection, the challenge protocol was carried out according to Ref. [36].

For zebrafish larvae immersion infections, zebrafish larvae at 5 day-post fertilization (dpf) were immersed with PBS containing 10⁸ CFU/ml *E. piscicida* for 2 h at 28 °C, then washed three times with E3 buffer for 5 min each time. Subsequently, zebrafish larvae were transferred to 10 cm dishes, with approximately 50 larvae in 15 ml E3 medium per dish, and incubated at 28 °C. The mortality rate was then observed at different time points.

2.6. Total RNA isolation, cDNA synthesis and RT-PCR

Total RNA was extracted from samples using Trizol (15596018, Life Technologies) according to the manufacturer's instruction, and extracted RNA was measured with NanoDrop ND 2000 (Thermo, USA). To remove residual genomic DNA, RNA samples were digested with RNase-free DNase I (Tiangen, China). Immediately, 1 mg total RNA was amplified in cDNA synthesis reactions by using PrimeScript RT reagent kit (TaKaRa, Japan). The reaction solution was mixed and incubated at 37 °C for 15 min, followed by heat inactivation at 85 °C for 5 s. Negative controls lacking reverse transcriptase or RNA were included for each group. The final cDNA reaction mixtures (20 µl) were diluted with 80 µl of water and stored at 20 °C until use.

RT-qPCR was carried out following the manufacturer's instruction of SYBR green real-time PCR mix (Tiangen, China) using ABI 7500 Real-time Detection System (Applied Biosystems, USA). All samples were analyzed by RT-qPCR in triplicate for technical replicate. A melting curve analysis was performed for all PCR products to confirm the

occurrence of specific amplification peaks and the absence of primer dimer formation. Primers for each gene were described in Table 1. The gene expression was evaluated for three biological replicates, and the data for each sample was determined by comparative threshold cycle method ($2^{-\Delta\Delta CT}$ method) with β -actin as reference gene.

2.7. Turbot vaccination and challenge

Turbots weighing 35.0 ± 5.0 g were obtained from a commercial fish farm (Tianyuan, Shandong, China) and acclimatized in our laboratory for two weeks before the experimental manipulation. Fish were reared in aerated tanks supplied with a continuous flow of sand-filtered seawater at 15.0 ± 1.0 °C.

For safety evaluation experiment, a group of 20 turbot were challenged with WED or WED Δ T6SS (6×10^7 CFU per fish) by intraperitoneal injection.

For efficacy evaluation experiment, a group of 150 turbot were immunized with WED or WED Δ T6SS (3×10^5 CFU per fish) by intraperitoneal injection, an additional group of 150 turbot were mock immunized with PBS. The fish were maintained in aerated free flowing freshwater for 30 days and then challenged by intramuscular injection of *E. piscicida* EIB202 (2×10^3 CFU per fish), then the cumulative mortalities were recorded over a 2-week period. Both vaccination and challenge were conducted in triplicate, and the cumulative survival rate was determined over a 2-week period.

Relative percent survival (RPS) was calculated according to the following formula:

$$\text{RPS} = (1 - \% \text{ mortality of immunized fish} / \% \text{ mortality of control fish}) \times 100\%$$

2.8. Statistical analysis

Statistical analysis was performed by Prism 7.0 (Graphpad, USA). Each experiment was performed at least three times with 3 parallel repeats. The statistical significance was determined by one-way ANOVA. In all cases, the significance level was defined as * $p \leq 0.05$, ** $p \leq 0.01$.

3. Results

3.1. T3SS and T6SS play different roles in *E. piscicida* infection of epithelial cells

Previous studies have revealed that *E. piscicida* could infect both epithelial [37] and phagocytic cells [38], and showed a replicative intracellular life style in these cells. But the specific role of T3SS and T6SS during infection is still not clear. In this study, we firstly explored the contributions of T3SS and T6SS to the different infection processes of *E. piscicida* in epithelial cells. At the early stage of infection, T3SS mutant and T6SS mutant did not affect the adhesion ability when compared to the wild-type EIB202 (Fig. 1A), indicating that T3SS and T6SS are not involved in bacterial adhesion. During bacterial internalization, T3SS mutant showed a lower ratio of internalized bacteria compared to EIB202, while T6SS mutant had a much higher ratio (Fig. 1B), suggesting that EIB202 T3SS contributes to bacterial invasion in HeLa cells, and T6SS conversely inhibits the bacterial internalization. In the later intracellular stage of infection, the intracellular replication ability of these three strains in HeLa cells were further determined. Intriguingly, the replication of T3SS mutant inside HeLa cells was completely abolished, while T6SS mutant showed higher proliferation efficiency than EIB202 (Fig. 1C), demonstrating that *E. piscicida* utilizes its T3SS to promote bacterial intracellular replication and T6SS to restrain the intracellular overload. Subsequently, we assessed the release of LDH at 5 h post-infection, and found a significantly reduced cytotoxicity in

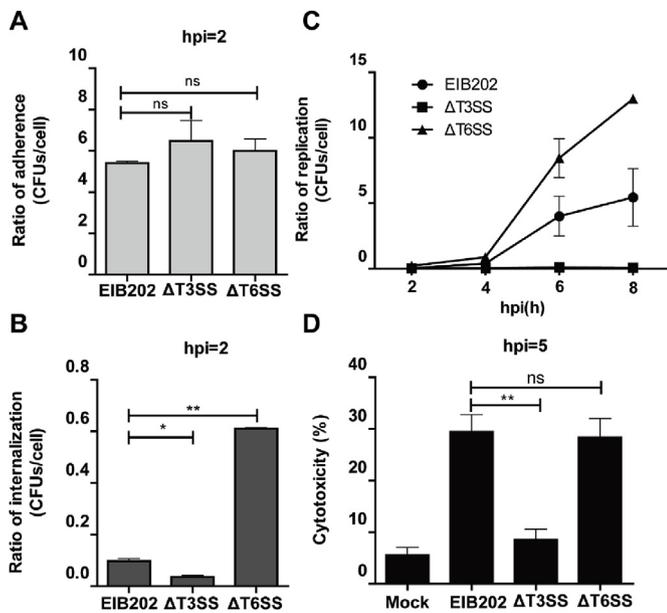


Fig. 1. T3SS and T6SS play different roles in *E. piscicida* infection of epithelial cells. HeLa cells were infected with EIB202, Δ T3SS, and Δ T6SS at an MOI of 100. (A) After 2 h post infection, the adherent bacteria was counted, and the results were expressed as the ratio (CFUs per cell). (B) At the indicated time, the ratio of internalization was examined. (C) Within 2 h, infected cells were treated with 1000 μ g/ml gentamicin for 30 min to kill extracellular bacteria. After incubation for 2 h, cells were cultured in growth medium containing 10 μ g/ml gentamicin for the indicated time-intervals. Triton X-100 was added to the cell culture at 1% (v/v) at the indicated hours post infection, the lysed mixture was serially diluted and counted by CFU, the results are shown as the ratio (CFUs per cell). (D) After 5 h post infection, cytotoxicity of infected cells was assessed as the release of lactate dehydrogenase. Graphs show the mean \pm SD of triplicate cultures, data are representative of at least 3 experiments. * p < 0.05, ** p < 0.01.

T3SS mutant-infected HeLa cells compared to T6SS mutant and EIB202 (Fig. 1D), suggesting that *E. piscicida* T3SS can trigger cell death in HeLa cells.

3.2. T3SS and T6SS play different roles in *E. piscicida* infection of macrophages

Invasion and intracellular survival in host macrophages was also reported to be important for *E. piscicida* systemic infection [39]. Therefore, we next investigated the roles of T3SS and T6SS during infection of J774A.1 cells with *E. piscicida*. Consistent with the results in HeLa cells, the absence of T3SS and T6SS had no effect on the adhesion of EIB202 to J774A.1 cells (Fig. 2A). Furthermore, there were no differences in bacterial internalization between EIB202, T3SS mutant, and T6SS mutant (Fig. 2B), suggesting that T3SS and T6SS are not involved in the internalization of EIB202 in J774A.1 cells. Interestingly, T3SS mutant showed an obvious defect in proliferation within J774A.1 cells, and T6SS mutant showed higher proliferation efficiency than EIB202 (Fig. 2C). Moreover, T6SS mutant induced more pronounced cytotoxicity than EIB202, while the absence of T3SS restricted the cytotoxicity in J774A.1 cells (Fig. 2D), suggesting that T3SS is responsible for inducing cytotoxicity, while T6SS help to suppress cytotoxicity in J774A.1 cells. Taken together, these data suggested that T3SS of *E. piscicida* is essential for intracellular replication and triggering cell death while T6SS is responsible for controlling the overload and cell death in macrophages.

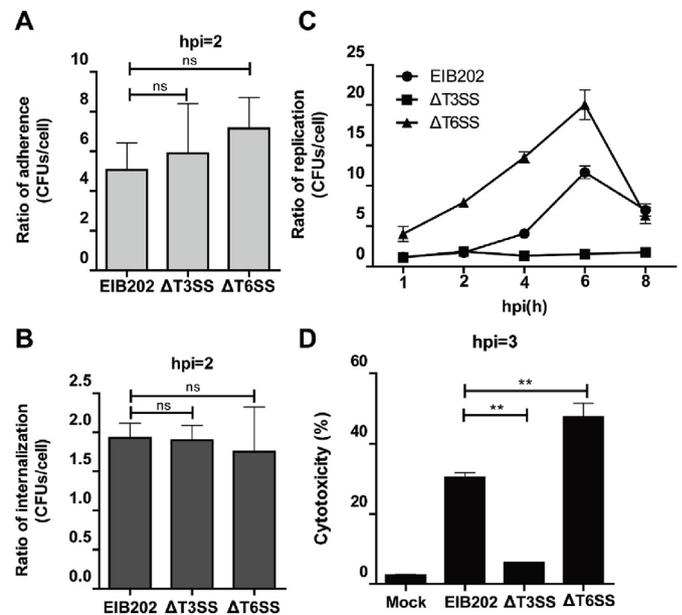
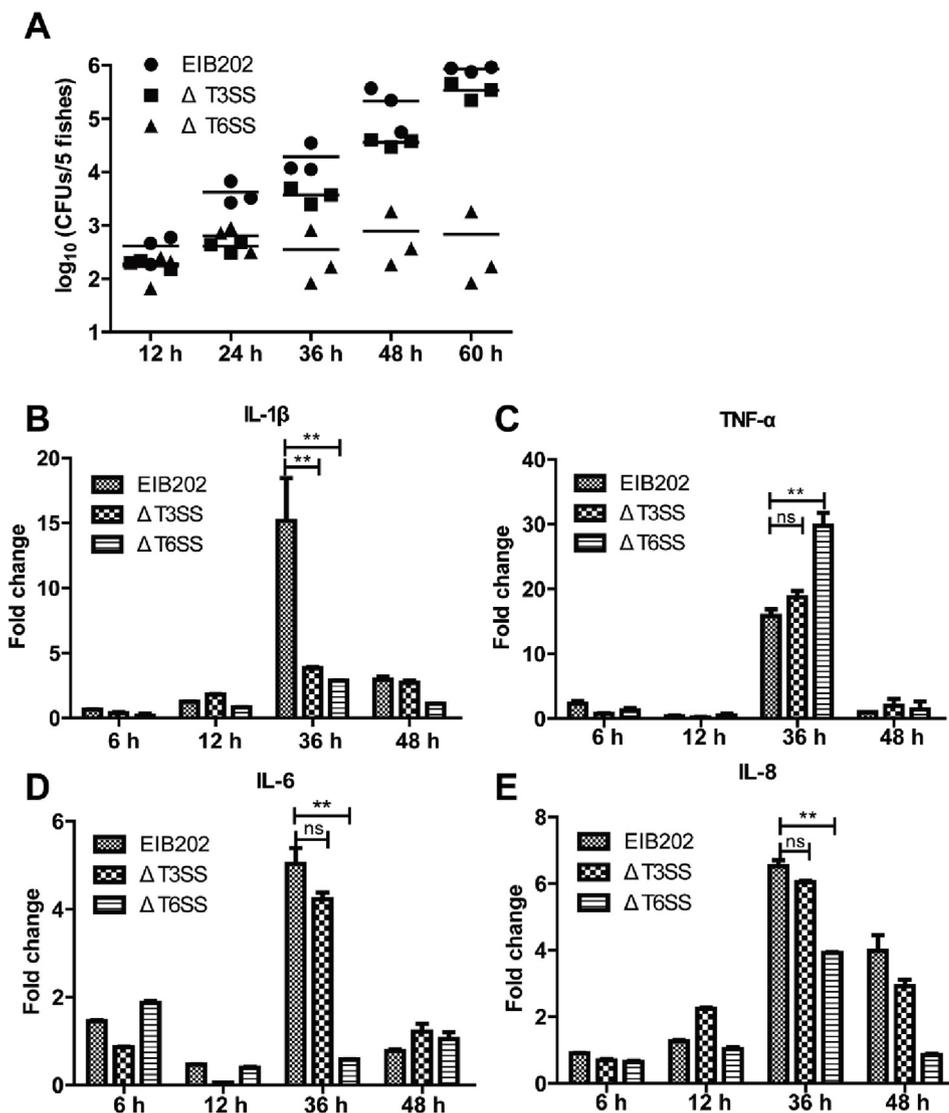


Fig. 2. T3SS and T6SS play different roles in *E. piscicida* infection of macrophage. J774 A.1 cells were infected with EIB202, Δ T3SS, and Δ T6SS at an MOI of 10. (A) After 2 h post infection, the adherent bacteria was counted, and the results were expressed as the ratio (CFUs per cell). (B) At the indicated time, the ratio of internalization was examined. (C) Within 2 h, infected cells were treated with 1000 μ g/ml gentamicin for 30 min to kill extracellular bacteria. After incubation for 2 h, cells were cultured in growth medium containing 10 μ g/ml gentamicin for the indicated time-intervals. Triton X-100 was added to the cell culture at 1% (v/v) at the indicated hours post infection, the lysed mixture was serially diluted and counted by CFU, the results are shown as the ratio (CFUs per cell). (D) After 3 h post infection, cytotoxicity of infected cells was assessed as the release of lactate dehydrogenase. Graphs show the mean \pm SD of triplicate cultures, data are representative of at least 3 experiments. * p < 0.05, ** p < 0.01.

3.3. T6SS contributes to regulate host immune response to facilitate *E. piscicida* infection in vivo

Previous studies have demonstrated that *E. piscicida* colonizes in various organs, such as the liver, spleen, and kidney [40]. Therefore, we wanted to assess the contributions of T3SS and T6SS to the colonization of *E. piscicida* in zebrafish. First of all, we examined the infection kinetics of EIB202 in different organs of zebrafish and indicated that EIB202 developed more bacterial CFUs in the kidney than in the liver and spleen (Fig. S1). Next, we compared the bacterial burden of EIB202, T3SS mutant, and T6SS mutant in the zebrafish kidney. As shown in Fig. 3A, T3SS mutant only had slightly weaker colonization than EIB202, but T6SS mutant reduced bacterial burden significantly in the kidney (Fig. 3A), indicating that T6SS is important for *E. piscicida* colonization in zebrafish.

When pathogens penetrate the external barriers of an organism and cause infection, a non-specific inflammatory response is triggered to clear the invading bacteria [41]. Considering the quick clearance of T6SS mutant during infection, we are wondering if T6SS of *E. piscicida* is responsible for evading host inflammatory response. Cytokines are particularly important and one of the most studied mediators of innate immunity response, such as interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-8 (IL-8) [42]. We thus explored the contributions of T3SS and T6SS to the transcription of inflammatory factors in zebrafish kidney. At 36 h post-infection, T6SS mutant showed decreased expression of IL-1 β , IL-6 and IL-8 which may be due to its lower bacteria load. Interestingly, compared with EIB202, T6SS mutant behaved an enhanced transcriptional expression of TNF- α even though it had a lower bacterial burden at this time point



(Fig. 3B–E). Meanwhile, T3SS mutant inhibited the transcription of IL-1 β at 36 h (Fig. 3B). Collectively, these results suggested that T6SS play important roles in inhibiting the transcription of TNF- α responses to facilitate *E. piscicida* infection *in vivo*.

3.4. T3SS and T6SS contribute together to the virulence of *E. piscicida* in zebrafish

Since the different roles of T3SS and T6SS during different infection processes we explored above, we further assessed the contributions of T3SS and T6SS in the infection of *E. piscicida* *in vivo*. Firstly, we infected adult zebrafish with EIB202, T3SS mutant, and T6SS mutant, and monitored fish survival after infection. During the 6-day experiment, 60% of fish in T3SS mutant-challenged group and 80% in T6SS mutant-challenged group survived, while only 20% of fish in the EIB202-challenged group survived (Fig. 4A). In addition, we also examined the pathogenicity of these strains in zebrafish larvae. Following immersion infection, zebrafish in EIB202-challenged group rapidly died, whereas the T3SS mutant-challenged group and the T6SS mutant-challenged group exhibited significantly high survival rate (Fig. 4B). Moreover, after microinjection of the yolk sac, the survival rate of zebrafish in the T3SS mutant-challenged group was about 80%, and the survival rate of zebrafish in the T6SS mutant-challenged group was 70%, while the survival rate of zebrafish in the EIB202-challenged group was only 20%

Fig. 3. T6SS contributes to regulate host immune response to facilitate *E. piscicida* infection *in vivo*. Zebrafish were challenged with EIB202, Δ T3SS, and Δ T6SS (100 CFU per fish), at indicated times, the kidneys of 5 fish were sampled. (A) The samples were processed for subsequent CFU counting, and the bacterial colonization was examined. (B)–(E) The samples were processed for qRT-PCR, the mRNA levels of each gene was normalized to that of β -actin and relative expression was calculated by dividing the values of the challenged tissues by those of the control. (B) IL-1 β . (C) TNF- α . (D) IL-6. (E) IL-8. Graphs show the mean \pm SD of triplicate cultures, data are representative of at least 3 experiments. * p < 0.05, ** p < 0.01.

(Fig. 4C). Taken together, these results suggested that T3SS and T6SS are required for the virulence of EIB202 in zebrafish together.

3.5. Optimization of vaccine with T6SS

Previous studies in our lab have constructed *E. piscicida* mutant strain WED, which derived from the whole genome sequenced EIB202 strain with disrupted T3SS, and demonstrated that the live attenuated *E. piscicida* vaccine strain WED showed efficacious protection in turbot [32]. However, subsequent experiments have shown that when the immune dose of WED is increased to 200 times, the biosafety of the vaccine is reduced (Fig. 5A). Because *E. piscicida* T6SS are required for bacterial virulence in zebrafish, based on WED, we constructed WED Δ T6SS strain, and as shown in Fig. 5A, at the same dose of immunization, the survival rate of turbot in WED Δ T6SS group was significantly higher than that in WED group, suggesting that the absence of T6SS could improve the biosafety of WED. We further tested whether the absence of T6SS affects the immune protection of WED. 2 weeks after challenged with EIB202, the RPS of fish vaccinated with WED Δ T6SS showed no significant difference with that vaccinated with WED (Fig. 5B), indicating that deletion of T6SS does not affect the immune protection of WED.

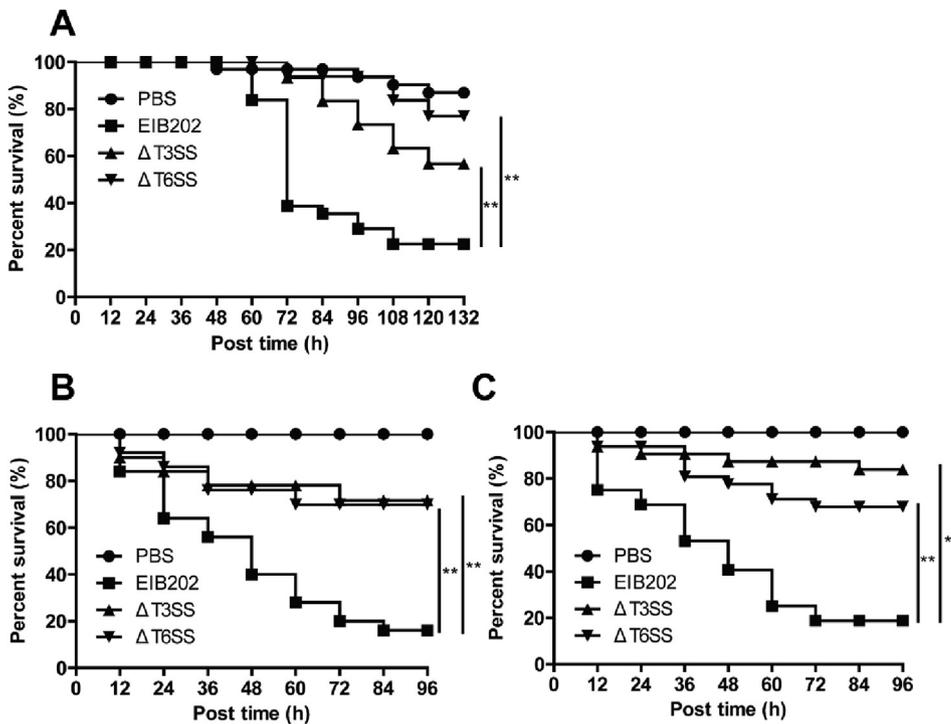


Fig. 4. T3SS and T6SS contribute together to the virulence of *E. piscicida* in zebrafish. (A) Adult zebrafish were challenged with PBS, EIB202, Δ T3SS, and Δ T6SS (100 CFU per fish) by intramuscular injection. Then the survival rate of zebrafish larvae was examined. (B) Zebrafish larvae of 5 dpf were infected by immersion with PBS, EIB202, Δ T3SS, and Δ T6SS (5×10^7 CFU/ml), then survival rate of zebrafish larvae was monitored for 4 days. (C) Zebrafish larvae of 3 dpf were infected with PBS, EIB202, Δ T3SS, and Δ T6SS (100 CFU per larvae) by microinjection, then survival rate of zebrafish larvae was monitored for 4 days. Similar results were obtained in two separate experiments. * $p < 0.05$, ** $p < 0.01$.

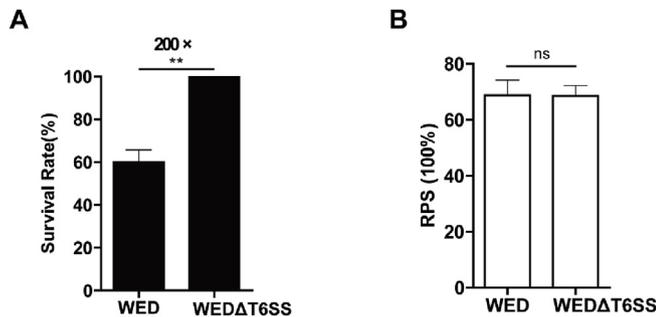


Fig. 5. Optimization of vaccine with T6SS. (A) Turbot fish were challenged with WED or WED Δ T6SS (6×10^7 CFU per fish) by intraperitoneal injection (20 fish per group). The experiments were performed in triplicate, and the mortalities were recorded for 15 days. (B) The immunized and PBS mock-immunized group were challenged with EIB202 (2×10^3 CFU per fish) 30 days after immunization. The experiments were performed in triplicate, and the mortalities were recorded for 15 days. Both vaccination and challenge were conducted in triplicate, graphs show the mean \pm SD of triplicate cultures, data are representative of at least 3 experiments. * $p < 0.05$, ** $p < 0.01$.

4. Discussion

Bacterial pathogens utilize a multitude of methods to invade mammalian hosts, damage tissue sites, and thwart immune system from responding. One essential strategy for many pathogens is the secretion of proteins across phospholipid membranes [1]. T3SS and T6SS were identified as two of the most important secretion systems in *E. piscicida* [11,12]. These two secretion pathways inject effectors directly into target cells, provide an advantage to pathogens in the hosts and indirectly contribute to bacterial colonization and persistence.

Yang et al. (2015) demonstrated that T3SS and T6SS mutants were inefficient to invade the macrophage, and they also proved that T3SS and T6SS mutants were unable to propagate in turbot organs [20]. In this study, we are in the effort to gain a further understanding of how T3SS and T6SS work together during the infection of *E. piscicida* *in vitro* and *in vivo*. First of all, we found a “balanced” role of T3SS and T6SS during *E. piscicida* infection. Specifically, T3SS is central for *E. piscicida*

intracellular life in host cells while T6SS is responsible for subverting the invasion and excessive replication in host cells (Figs. 1 and 2). Compared with Yang [20], this study firstly revealed the balance between T3SS and T6SS. The roles of T3SS in bacterial invasion and intracellular survival has been widely demonstrated in other Gram-negative pathogens like *E. coli* [7,8] and *Salmonella* [3,4]. Meanwhile, the role of T6SS in controlling bacterial virulence during infection is also noteworthy. The *Helicobacter hepaticus* T6SS was shown to limit its colonization within intestinal epithelial cells by promoting a balanced relationship between microbe and host [43]. We suspected that such a “balanced” role of T6SS is important for long-term colonization since robust infection could trigger intensive host immune defense.

Secondly, we found a bilateral role of T3SS and T6SS which respectively act as spear and shield during *E. piscicida* infection. When *E. piscicida* penetrates host epithelial barrier and macrophage cells, and colonizes within these cells as a replicative niche, it activates the innate immune system which can obviously result in pathogen clearance, and this is likely leading to the acquisition of T6SS to suppress innate immune signaling pathways. Indeed, T3SS of *E. piscicida* was reported to activate NLRC4 and NLRP3 inflammasomes while a T6SS effector EvpP was found to inhibit NLRP3 inflammasome activation [17]. Consistent with these conclusions, here, we found that the T3SS mutant restrained cell death (Figs. 1D and 2D) and the T6SS mutant induced more cell death (Fig. 2D). However, the amount of bacteria may also be involved in this process, as the T3SS mutant showed less bacterial load, and the T6SS mutant had higher bacterial load than EIB202 (Fig. 2B).

Inflammatory cytokines and chemokines play critical roles in mediating host inflammation responses during bacterial infection [44]. In response to infection, TNF- α is a primary proinflammatory mediator involved in immune response [45], which could work as a regulator for neutrophils ROS generation [46]. In addition, the inflammasome activation induced a robust IL-1 β production, which could promote the recruitment of neutrophils and ROS release to kill the pathogens [47]. Previous study has revealed that *E. piscicida* infection could induce a robust expression of TNF- α and IL-1 β in zebrafish larvae, which suggest that the inflammasome and MAPK pathways might be activated in response to infection in zebrafish [48]. In this study, we found that T3SS of *E. piscicida* can increase the expression of IL-1 β *in vivo* while T6SS can

overcome the innate immune response, especially the TNF- α expression to establish infection, these results complemented the mechanism by which *E. piscicida* activates inflammatory cytokines (Fig. 3B–E). Remarkably, both T3SS and T6SS are pivotal for *E. piscicida* virulence *in vivo* (Fig. 4), suggesting their cooperative roles during *E. piscicida* infection.

E. piscicida is the etiologic agent of edwardsiellosis, a devastating fish disease prevailing in worldwide aquaculture industry [12], and vaccination is one of the strategies for preventing disease through stimulating host immune response. So far, different types of vaccine including formalin-killed vaccine [33], live attenuated vaccine [32], subunit vaccine [34] and DNA vaccine [35] have been attempted against *E. piscicida*. The effect of virulence modification is always the first concern in development of vaccines. As a live vaccine, using with low immunization doses, WED showed good immune protection and high biosafety [32], however, we found that there is a problem with the biosafety of WED when we increased the immune dose (Fig. 5A). Based on our results, we optimized WED by combining WED with T6SS and constructed WED Δ T6SS. The optimized vaccine showed similar immune protection with WED in turbot (Fig. 5B), but it greatly enhanced the biosafety of the vaccine (Fig. 5A), developing a new promising vaccine candidate against *E. piscicida*. Moreover, further vaccine candidates combined of T3SS and T6SS effectors may be considered.

Our results supplement evidences indicating that the pathogenicity of *E. piscicida* mainly depends on functional T3SS and T6SS. In addition, we clarified the specific roles of T3SS and T6SS in *E. piscicida* infections and their relative contribution in pathogenesis of *E. piscicida*. These results provide a foundation for further studies of the pathogenesis of *E. piscicida* and revealed new targets for therapies against *E. piscicida*.

Acknowledgments

This work was supported by the NSFC grants 31472308 and 31622059 to Q.L., and The Fundamental Research Funds for The Central Universities.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.fsi.2019.08.014>.

Author contribution statement

Q.L., T.H., X.L., and D.Y. conceived the study; T.H. performed the majority of experiments. Z.W. participated in zebrafish larvae experiments. R.C. and L.Z. participated in J774A.1 cells experiments. Q.L., and T.H. analyzed all data and wrote the manuscript; Q.L., X.L., and Y.Z. critically revised the manuscript. All authors discussed the results and commented on the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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